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**Whole genome sequencing of 1058 *Enterococcus faecium* from Copenhagen, Denmark, reveals rapid clonal expansion of vancomycin-resistant clone ST80 combined with widespread dissemination of a *vanA*-containing plasmid and acquisition of a heterogeneous accessory genome**

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# Contributed equally

Running title: VREfm expansion and acquisition of accessory genes

36     **Abstract**

37     **Objectives:** From 2012-2015, a sudden huge increase of vancomycin-resistant (*vanA*) *Enterococcus*  
38     *faecium* (VREfm) was observed in the Capital Region of Denmark. Clonal relatedness of VREfm and  
39     vancomycin-susceptible *E. faecium* (VSEfm) was investigated, transmission events between hospitals  
40     were identified and the pan-genome and plasmids from the largest VREfm clonal group were  
41     characterized.

42     **Methods:** WGS of 1058 *E. faecium* isolates was carried out on the Illumina platform to perform SNP-  
43     analysis and to identify the pan-genome. One isolate was also sequenced on the PacBio platform to close  
44     the genome. Epidemiological data were collected from laboratory information systems.

45     **Results:** Phylogeny of 892 VREfm and 166 VSEfm revealed a polyclonal structure with a single clonal  
46     group (ST80) accounting for 40% of the VREfm isolates. VREfm and VSEfm co-occurred within many  
47     clonal groups; however, no VSEfm were related to the dominant VREfm group. A similar *vanA* plasmid  
48     was identified in  $\geq 99\%$  of isolates belonging to the dominant group and 69% of the remaining VREfm.  
49     Ten plasmids were identified in the completed genome and approximately 29% of this genome consisted  
50     of dispensable accessory genes. The size of the pan-genome among isolates in the dominant group was  
51     5,905 genes.

52     **Conclusions:** Most likely, VREfm emerged due to import of a successful VREfm clone which has  
53     rapidly transmitted to the majority of hospitals in the region whilst simultaneously disseminating a *vanA*  
54     plasmid to pre-existing VSEfm. Acquisition of a heterogeneous accessory genome may account for the  
55     success of this clone by facilitating adaption to new environmental challenges.

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## Introduction

*Enterococcus faecium*, a commensal in the gut, has emerged as an increasingly important nosocomial pathogen causing bacteraemia, intra-abdominal, urinary tract and intravenous -catheter related infections.<sup>1</sup> Acquired resistance to ampicillin, gentamicin (high-level) and vancomycin has increased worldwide among hospital-associated *E. faecium* narrowing treatment options of enterococcal infections.<sup>2-5</sup> Patients, asymptotically colonized with vancomycin-resistant *E. faecium* (VREfm) in the GI-tract act as both a reservoir and a source for dissemination of VREfm into the hospital environment.<sup>6</sup> This makes infection control of this problem pathogen highly challenging. Previous studies have identified two to ten asymptomatic VREfm carriers for each patient with a clinical VREfm isolate.<sup>6</sup>

Vancomycin is first line treatment of infections caused by *E. faecium*. Therefore, it was of great concern when the incidence of *vanA* VREfm started to increase in the Capital Region of Denmark in 2012.<sup>7</sup> More than 1,500 VREfm patients were identified from 2012 to 2015, contrasting with only 9 patients infected or colonised in 2011.<sup>8,9</sup>

We recently reported on WGS of 495 VREfm isolates identified from the Capital Region of Denmark from 2012-2014 and observed a polyclonal outbreak characterized by the spread of a highly successful plasmid (pHvH-V24, KX574671.1), which conferred resistance to vancomycin through the presence of a *vanA* gene complex.<sup>10</sup> This *vanA* plasmid had been acquired by >90% of the VREfm clonal groups. Therefore, we developed the hypothesis that a VREfm clone, containing a *vanA* plasmid, has been introduced to the Capital Region of Denmark and the plasmid was disseminated via horizontal gene transfer to pre-existing hospital-adapted vancomycin-susceptible *E. faecium* (VSEfm) clonal groups.

Hospital-associated *E. faecium* are known to rapidly acquire mobile genetic elements (MGE) and, as a result, they harbour plasmids and insertion elements that confer resistance, virulence and other clinically relevant phenotypes.<sup>11,12</sup> More detailed molecular data on MGEs, the accessory genome and comparison to VSEfm are therefore necessary to understand how VREfm and resistance determinants disseminate in the hospital environment.

Here, we applied short- and long-read sequencing along with epidemiological data to understand the sudden increase of VREfm in the low prevalence Capital Region of Denmark from 2012-2015. The aims of the study were 1) to analyse the genetic relatedness between temporally and geographically matched VREfm and VSEfm and to detect the spread of the *vanA* plasmid between different clones, 2) to identify VREfm transmission events between hospitals and 3) to characterize the pan-genome and plasmids within the largest clonal group accounting for 40% of the VREfm isolates.

## Methods

### *Setting and bacterial isolates*

105 The study was conducted in the Capital Region of Denmark (1.7 million inhabitants) from January 2012  
106 to December 2015. The healthcare system is served by 11 hospitals (one tertiary hospital, four large acute  
107 care secondary hospitals and six minor secondary hospitals). VREfm isolates from all Departments of  
108 Clinical Microbiology (n=3) were submitted consecutively for WGS from 2012-March 2014 (n=338)  
109 whereas only isolates from the Southern part of the region were submitted from April 2014-2015 (n=554).  
110 VREfm screening isolates were identified using selective chromogenic medium and MALDI-TOF.  
111 VREfm in clinical samples were identified using MALDI-TOF and vancomycin disc diffusion according  
112 to EUCAST.<sup>13</sup> Only *vanA* VREfm were included as the vast majority of the isolates contained that  
113 genotype (five isolates contained *vanB*). One isolate per patient was included.

114 VSEfm blood isolates from 2013-2014 (n=113) and VSEfm non-blood isolates from 2013-  
115 2015 (n=53) were included in the study. Ampicillin resistance was determined using disc diffusion  
116 according to EUCAST.<sup>13</sup>

117 Date of sampling, submitting hospital, hospital ward, sample site and sample type (clinical  
118 or screening sample) were collected for all isolates from the laboratory information systems.

119 VREfm identified from 2012-2014 were sequenced in a previous study.<sup>10</sup> All VREfm from  
120 2015 (n=399) and VSEfm (n=166) were sequenced for this study.

121

## 122 *Whole genome short read sequencing and comparative analysis*

123 BDNA extraction and sequencing using Illumina technology were conducted as previously described.<sup>7, 10</sup>  
124 Sequence reads were mapped to the V24 reference genome (see below) and to individual plasmids using  
125 SMALT.<sup>14</sup> Reads at indel sites were realigned using the GATK toolbox.<sup>15</sup> SNPs were called using  
126 samtools.<sup>16</sup> The variant call file (VCF) was filtered for variants supported by a minimum read depth of 4  
127 (minimum 2 per strand), >30 map quality, >50 average base quality, no significant strand bias and >75%  
128 of reads supporting the variant. Indels were additionally confirmed using pindel.<sup>17</sup> Repeat regions of  
129 >50bp and any regions <100 bp between two repeat regions, which are notoriously problematic for short  
130 read mapping, were identified using nucmer and removed from the VCF resulting in consensus  
131 sequences.<sup>18</sup>

132 A phylogenetic tree (neighbour-joining tree) including all VREfm and VSEfm isolates was  
133 constructed using RapidNJ and tree visualization was done using FigTree.<sup>19, 20</sup> In addition, a subtree of  
134 isolates belonging to the largest clonal group was constructed using RAxML using the general time  
135 reversible model and gamma model of rate heterogeneity. Recombination regions were identified and  
136 removed using ClonalFrameML with a kappa setting of 4, and priors of  $R/\theta = 0.462$ ,  $1/\delta = 0.004$ ,  
137  $\nu = 0.042$ , allowing the rate of recombination to vary per branch.<sup>21, 22</sup>

138 MLST was determined *in silico* using pubMLST.

139

140 *Generating a complete genome sequence for a vanA E. faecium isolate using hybrid sequencing*  
141 A screening isolate, V24, which was an early representative of the largest VREfm clonal group, was  
142 identified as a reference strain. DNA extraction, Illumina sequencing and PacBio sequencing of V24 were  
143 performed as described previously.<sup>7, 10</sup> We achieved a total of 241,971 PacBio reads with a mean length  
144 of 5.8 kb.

145 V24 was assembled using a hybrid assembly methodology using both short, low error rate  
146 Illumina reads and PacBio long-reads. Reads were assembled by SPAdes v3.6.2 using the --careful, --  
147 pacbio, and a manual range of k-mer sizes of 21, 33, 55, 67, 77, 87, 97, 107, 117 and 127.<sup>23</sup> Contigs were  
148 scaffolded using SSPACE-longRead,<sup>24</sup> and gap filling was performed using PBJelly.<sup>25</sup> A single  
149 chromosome and ten non-chromosomal contigs/scaffolds >600 bp were assembled. The chromosome was  
150 found to be largely contiguous with previously published reference genomes after alignment using  
151 MAUVE.<sup>26-28</sup> The ten non-chromosomal contig/scaffold sequences were searched against the NCBI nr/nt  
152 database using default megablast settings. Contigs and scaffolds were circularized using an in-house  
153 script as described previously.<sup>10</sup> All but one contig were circularisable using this methodology. PCRs  
154 were performed to circularize the last contig, but despite repeated attempts, this did not succeed.

155  
156 *Characterization of the complete genome V24*

157 The chromosome and all plasmids were annotated using RAST.<sup>29</sup> Resistance genes,  
158 virulence genes, prophages and plasmid replicons were identified via Antibiotic Resistance Gene-  
159 ANNOTation, virulenceFinder, Phast and PlasmidFinder online tools, respectively.<sup>30-32</sup> Plasmids were  
160 searched for Toxin-antitoxin systems (axe-txe and  $\omega$ - $\epsilon$ - $\zeta$ ) via BLAST according to Rosvoll *et al.*<sup>33</sup>  
161 Finally, copy number of plasmids was determined as the mean depth coverage (excluding repeat regions)  
162 of the plasmid divided by the mean depth coverage of the chromosome.

163  
164 *Presence of ten plasmids in VREfm and VSEfm isolates*

165 Illumina reads from all VREfm and VSEfm isolates were mapped to the ten plasmid sequences generated  
166 from the reference isolate, V24. The depth of coverage, breadth of coverage and the total number of SNPs  
167 (excluding repeat regions) was determined. A plasmid was characterized as present in a sample when at  
168 least 85% of the reference sequence was covered by sufficient reads to call a reference or variant base and  
169 the number of SNPs was  $\leq 5$  SNPs per 1000 bp (~99.5% similarity). A reduced breadth of coverage of  
170  $\geq 80\%$  was accepted for plasmid pV24-2 due to large amounts of repeat regions which led to lower  
171 breadth of read coverage (86%) even when mapping the short reads used to create the reference genome,  
172 V24, against itself.

173  
174 *Pan-genome analysis*

175 Reads were assembled *de novo* using Velvet v1.0.11 and contigs were annotated by Prokka v1.11. The  
176 pan-genome of the largest clonal group was identified by Roary using default settings.<sup>34, 35</sup> Core genes  
177 were defined as those present in 99% of the isolates with a minimum percentage amino-acid identity of  
178 95%. We excluded 27 out of 361 isolates (7.5%) from the pan-genome analysis due to poor assemblies  
179 (assemblies of  $\geq 800$  contigs).

180

#### 181 *Ethics*

182 The project has been approved by the Danish Data Protection Agency (2012-58-0004/AHH-2015-047)  
183 and the Danish Health and Medicines Authority (3-3013-1118/1).

184

### 185 **Results**

#### 186 *Descriptive data*

187 In total, 1,058 *E. faecium* isolates (892 *vanA* VREfm and 166 ampicillin resistant VSEfm) were included  
188 in the study. A third (n=292, 33%) of the VREfm isolates were from clinical samples and of these isolates  
189 12% (n=35) were from blood cultures. The vast majority of the VREfm isolates (n=832, 93%) were  
190 identified in the five largest hospitals in the region and most VSEfm isolates (n=156, 94%) were  
191 identified in the same five hospitals. More epidemiological data are presented in Table S1.

192

#### 193 *Population structure of VREfm*

194 SNP-based phylogeny revealed high diversity between hospital-associated VREfm isolates (Figure 1A).  
195 The isolates were separated into 16 main groups of  $\geq 3$  isolates, 10 minor groups of 2 isolates and 25  
196 singletons. The majority, n=728 (81%) of the isolates belong to five groups consisting of 47-361 VREfm  
197 isolates. Clonal group 2\_ST80 was the dominant clone from 2012-2015 (Figure 1B). Excluding group  
198 2\_ST80, the dominant clonal groups were 1\_ST117 and 3\_ST192 in 2012-2013 until they were replaced  
199 by clonal groups 8\_ST203 and 13\_ST80 in 2014-2015.

200

#### 201 *Comparative analysis of temporally and geographically matched VREfm and VSEfm*

202 The phylogeny showed that vancomycin-resistant and -susceptible isolates co-occurred within many of  
203 the clonal groups (Figure 1A). In total, n=146 (88%) VSEfm clustered together with VREfm. However,  
204 the largest VREfm group (2\_ST80, n=361), responsible for the first local outbreak of VREfm, contained  
205 no VSEfm. Also, clonal groups 8\_ST203 and 13\_ST80, which were prevalent from 2015, only contained  
206 1 and 0 VSEfm, respectively.

207

#### 208 *Epidemiology and spread of clonal group 2\_ST80*

209 The largest clonal group, 2\_ST80, was analysed further in order to understand the rapid dissemination  
210 throughout the region. A maximum likelihood phylogenetic tree, generated for group 2\_ST80, was  
211 annotated with hospital location and sampling date (Figure 2A). Further details are presented interactively  
212 using Microreact (<https://microreact.org/project/BkNqA1EDX>, use Google Chrome as browser).<sup>36</sup> Only  
213 samples from 2012 until March 2014 were included (n=142) because sampling coverage was uniform  
214 across the whole region for that time period. Very few SNP differences (mean pairwise SNPs = 8) were  
215 observed between the isolates. The epidemic curve shows that 90% (n=9) of the isolates in 2012 were  
216 sampled from Hospital 1, suggesting that Hospital 1 was the site into which group 2\_ST80 was  
217 introduced (Figure 2B). Subsequently, the clone spread to five more hospitals within six months showing  
218 that clonal group 2\_ST80 became widespread in the region within less than a year. The phylogenetic tree  
219 branches out in four subgroups. One branch is almost unique to Hospital 2 and the three other branches  
220 contain isolates from four or five hospitals. Assuming that each branch corresponds to a new introduction,  
221 this suggests that clonal group 2\_ST80 has been transmitted several times between hospitals in the region  
222 (frequent cross-hospital transmission).

223

#### 224 *Genome summary of V24*

225 A representative isolate, V24, of clonal group 2\_ST80, was selected for PacBio sequencing to investigate  
226 this successful group further. The genome of V24 included a single circular chromosome (2.7 Mb and  
227 2,625 coding sequences (CDS)), nine circular plasmids with sizes ranging from 4,304 bp to 172,811 bp  
228 (4-178 CDS) and one partial or linear plasmid of 103,806 bp (133 CDS) (Table 1). In total, the genome  
229 contained 3,139 CDS and approximately 14% of the genome was located on plasmids. The best hit in a  
230 blast search against the NCBI nr/nt database for each plasmid were previously published *E. faecium*  
231 plasmids with query coverages of >60% and similarity of >90%. The exception was the linear/partial  
232 plasmid, pV24-2, that was novel, with query coverage of 15% for the best hit.

233 The *vanA* plasmid, pV24-5, was closed in a previous study.<sup>10</sup> Re-analyses of PacBio and  
234 Illumina data in this study have extended the plasmid with 4,016 bp (Figure S1). This extension was  
235 confirmed by PCR. The added sequence contained four hypothetical proteins and a replication protein. The  
236 plasmid contained several resistance genes and a BLAST search revealed that the plasmid was similarly  
237 to the non-conjugative plasmid pS177 (Table 1).<sup>37</sup> Compared to the Tn1546 prototype characterized by  
238 Arthur *et al*,<sup>38</sup> the *vanA* transposon was truncated with a deletion of the transposase and 156 bp of the  
239 resolvase. An insertion element (IS1251) was inserted in the *vanSH* intergenic region. The loss of the  
240 transposase suggests that the element is no longer mobile and genes involved in the mobilization of the  
241 plasmid could not be identified.



Genes responsible for conjugation and horizontal transfer (*traG*) were identified in two other plasmids, pV24-1 and pV24-3 (Table 1),<sup>39</sup> and a BLAST search revealed that pV24-3 was similar to a highly conjugative plasmid pZB18.<sup>40</sup>

A search for prophages identified both intact and incomplete prophage regions in the chromosome and four plasmids. Importantly, the linear/partial plasmid, pV24-2, had three incomplete prophage regions of which many coding sequences corresponded to sequences in a linear bacteriophage recently described in a closed *E. faecalis* genome.<sup>41</sup> This may explain why pV24-2 was not circularisable.

#### *Distribution of V24 plasmids among the E. faecium population*

V24 plasmids (pV24-1- pV24-10) were much more frequent in VREfm than in VSEfm isolates (Table 2). Within group 2\_ST80 to which V24 belongs, the prevalence of V24 plasmids was highly variable. Two plasmids were present in <10% of the isolates and four plasmids were present in >90% of the isolates. This pattern shows that isolates belonging to the same clonal group contain different large accessory elements. The conjugative plasmid, pV24-3, was only present in group 2\_ST80 isolates identified from 2012-13. After 2013, the plasmid disappeared.

The *vanA* plasmid (pV24-5) was frequent, n=727 (81%) and spread across all but one VREfm clonal group (Group 8\_ST203). The remaining V24 plasmids were also rare in group 8\_ST203 indicating that there has been no exchange of plasmids between the two largest VREfm clonal groups.

#### *Pan-genome analysis*

The pan-genome of group 2\_ST80 was identified to assess the distribution between core genes and accessory genes within a clonal group of highly similar isolates determined by SNP analysis (mean pairwise SNPs = 8). The pan-genome contained 5,905 genes of which 1,684 were core genes, 2,223 were soft core genes (present in  $\geq 95\%$ ) and 2960 were cloud genes (present in <15%) (Figure 3). We know that the V24 isolate, belonging to group 2\_ST80, contained 3,139 CDS. A calculation shows that approx. 71% (2,223 soft core genes/3,139 CDS) of the V24 genome consisted of conserved and essential soft core genes and the remaining 29% of the genome consisted of non-essential accessory genes.

## **Discussion**

This study provides insights into a polyclonal VREfm outbreak which arose in the Capital Region of Denmark in 2012. The largest clonal group (2\_ST80) accounting for 40% of the isolates was responsible for the first local VREfm hospital outbreak. This was followed by a rapid clonal expansion and transmission into multiple hospitals in the region. No VSEfm isolates clustered in this group despite the large number of isolates sampled, so it can be assumed that clonal group 2\_ST80 was imported to the region as a vancomycin-resistant clone in 2012. A *vanA* plasmid, pV24-5, initially identified in a group

277 2\_ST80 isolate (V24) was observed in 81% of all VREfm isolates (Table 2). We also observed that  
278 VSEfm and VREfm co-occurred in many clonal groups which supports the hypothesis that concurrently  
279 with clonal expansion, group 2\_ST80 successfully disseminated pV24-5 via HGT to pre-existing  
280 hospital-adapted VSEfm and new VREfm clones were generated.

281 Group 2\_ST80 spread rapidly (within 6-9 months) to multiple hospitals within the Capital  
282 Region after its introduction to hospital 1 (Figure 2). This remarkable fast transmission is likely a product  
283 of the highly interlinked healthcare network within the region, with medical specializations consolidated  
284 into only a few Hospitals, which results in frequent transfer of patients between hospitals (approximately  
285 1,500 patients per month between hospitals, data from Department of Financial Affairs, the Capital  
286 Region of Denmark). VREfm colonized patients are asymptomatic carriers, and transfer of those patients  
287 represents a great risk of starting a new VREfm outbreak. In previous studies, clonally related VREfm  
288 isolates were identified in hospitals that rarely share patients and a community reservoir was suggested.<sup>27,</sup>  
289 <sup>28, 42-46</sup> In contrast, our genomic analyses and epidemiological data support a hospital reservoir for the  
290 dominant clonal group (2\_ST80).

291 From the beginning of the VREfm outbreak, barrier precautions, extra cleaning,  
292 disinfection with chlorine and VREfm screening of contact patients were initiated. Active surveillance  
293 screening programmes were not systematically introduced at the same time in all hospitals in the region  
294 which may explain continued expansion of VREfm.

295 The increase of VREfm and results from the present study prompted several measures to  
296 control VREfm: 1) Switch from a culture based methods to *vanA/vanB* PCR to achieve faster results (<24  
297 hours) and higher sensitivity, 2) Active surveillance screenings of wards with  $\geq 2$  VREfm patients, 3)  
298 VREfm screening of patients transferred from another hospital to high risk departments, 4) Screening and  
299 isolation of VREfm patients readmitted within six months and 5) real-time sequencing of VREfm isolates  
300 (results within 1-2 weeks) to include/exclude patients from outbreaks and to better understand  
301 transmission routes.

302 In 2015, clonal group 8\_ST203 emerged (Figure 1). This group did not contain the  
303 successful *vanA* plasmid, pV24-5, and only one VSEfm clustered in the group suggesting import and  
304 spread of yet another successful VREfm clonal group. Recently, the Danish Reference Laboratory at  
305 Statens Serum Institut closed a genome of an early (October 2014) group 8\_ST203 isolate.<sup>42, 47</sup> A *vanA*  
306 plasmid, pVRE1589 (CP020486), different from pV24-5 (Figure S1), was identified. Ninety-seven  
307 percent of the isolates in clonal group 8\_ST203 contained pVRE1589 (Henrik Hasman, Department of  
308 Bacteria, Parasites and Fungi, Statens Serum Institut, personal communication). Introduction of a clonal  
309 group containing a new *vanA* plasmid indicates that the hospital environment in the Capital Region has  
310 become favorable for VREfm within the last five years, as the prevalence of VREfm was less than 20  
311 isolates per year before 2012.<sup>8, 9</sup> Important factors to drive the current emergence of VREfm could be

312 increasing use of vancomycin and metronidazole due to a large *Clostridium difficile* outbreak, shift of  
313 empirical therapy from cefuroxime to piperacillin-tazobactam and increasing tolerance for disinfectants  
314 among *E. faecium* isolates.<sup>48-52</sup>

315 WGS-based typing is an important tool for infection control to define nosocomial  
316 outbreaks, include/exclude patients from an outbreak and to identify transmission events. In this work we  
317 identified widespread dissemination of a *vanA*-containing plasmid. Short read sequencing allowed for  
318 cost-effective epidemiological analysis and isolate screening on a large scale. Long-read sequencing  
319 allowed us to resolve problematic regions of the genome, such as plasmids, which were relevant to the  
320 current work. Based on this study we recommend using a combination of long-read and short-read  
321 sequencing for bacteria with highly variable accessory genome content to interpret outbreaks correct.

322 The present study identified a large number of MGEs in VREfm with 14% of the coding  
323 capacity located on plasmids in V24. This was in line with the pan-genome analysis of group 2\_ST80  
324 which identified acquisition of a highly heterogeneous accessory genome (Figure 3) suggesting frequent  
325 flux of MGEs. This flexible and mobile genome may contribute to the success of the clone by providing it  
326 with ready access to genes which enable it to adapt to new environmental changes. Lack of the CRISPR-  
327 Cas system, which provides bacteria with sequence-specific, acquired defense against plasmids and  
328 phage, was suggested as an explanation for the high number of MGEs in enterococci.<sup>53</sup> Previous studies  
329 have reported up to six plasmids per *E. faecium* genome with 9-10% of the genome located on  
330 plasmids.<sup>27, 28, 42-44</sup> Potential donors to the heterogeneous accessory genome could be other intestinal  
331 bacteria as *E. faecium* is part of a complex community in the GI-tract. However, previous studies showed  
332 that sequence importation in relation to recombination was mainly from other *E. faecium* populations.<sup>54, 55</sup>

333 The *vanA* plasmid, pV24-5, was highly similar to plasmid pS177, which was identified in a  
334 VREfm isolate from USA more than ten years ago and recently a pS177-like plasmid was reported in  
335 Australia, suggesting that the plasmid is globally spread.<sup>37, 44</sup> The plasmid, pS177, is a non-conjugative  
336 plasmid lacking the transposase in Tn1546 which indicates that the element is not mobile.<sup>37</sup> Presumably,  
337 the *vanA* plasmid, pV24-5, used the conjugation machinery from another conjugative plasmid to  
338 disseminate via HGT. Adding PacBio sequencing to close the genome of V24 revealed a conjugative  
339 plasmid, pV24-3, which was present in all group 2\_ST80 isolates identified from 2012 until September  
340 2013 (Table 2). This suggests that pV24-3 may have played an important role in the horizontal transfer of  
341 the *vanA* plasmid. From 2014, pV24-3 disappeared from the clone and was either replaced by another  
342 conjugative plasmid or the ability to disseminate the *vanA* plasmid was lost. This may explain why clonal  
343 groups 1\_ST117 and 3\_ST192 almost disappeared in 2015 (Figure 1). However, the *vanA* plasmid, pV24-  
344 5 was also present in clonal group 13\_ST80 which became quiet dominant in 2015. Our knowledge of  
345 conjugative transfer systems and plasmid interactions is sparse and hopefully, long-read sequencing will

346 improve our understanding of this. Also, experimental characterization of the required attributes for  
347 mobility and other accessory genetic elements contributing to this would be valuable.

348           The strength of this study is the large number of contemporary VREfm and VSEfm  
349 isolates that are included. Inclusion of VSEfm data, which is new compared to our previous study on  
350 VREfm has provided valuable information on the close relationship between VREfm and VSEfm.<sup>10</sup>  
351 Completion of a *vanA* genome enabled us to assess the plasmid content and provided us with a reference  
352 genome closely related to the outbreak we are investigating. However, this study has limitations. We  
353 expect that the plasmid population is much larger than investigated in this study. In addition, mixed  
354 infections may have been overlooked due to the sampling methodology of selecting only one colony for  
355 sequencing from each patient.

356           In conclusion, we have shown that a successful VREfm clone was imported to a VREfm  
357 low prevalence region in Denmark in 2012. Both clonal expansion and generation of new VREfm clonal  
358 groups via horizontal transfer of a *vanA* plasmid to pre-existing VSEfm contributed to the sudden increase  
359 and persistence of VREfm. This combination of clonal spread and plasmid spread must be recognized to  
360 interpret VREfm hospital outbreaks correctly and highlights the added value of long-read sequencing in  
361 WGS.

362           The acquisition of a highly heterogeneous accessory genome and the high number of  
363 plasmids within group 2\_ST80 showed that there is no single successful combination of accessory genes,  
364 suggesting that VREfm isolates belonging to this clone can rapidly acquire genes to optimize survival in  
365 the harsh hospital environment between transmissions.

366

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375

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379

## 380 **Transparency declarations**

381     None to declare.

382

383     **Accession numbers**

384     Illumina sequence data for all isolates (VREfm and VSEfm) were deposited in the ENA under the  
385     projects PRJEB14625 and PRJEB28731. The closed genome sequences of V24 were deposited at  
386     Genbank under accession numbers CP036151-CP036161.

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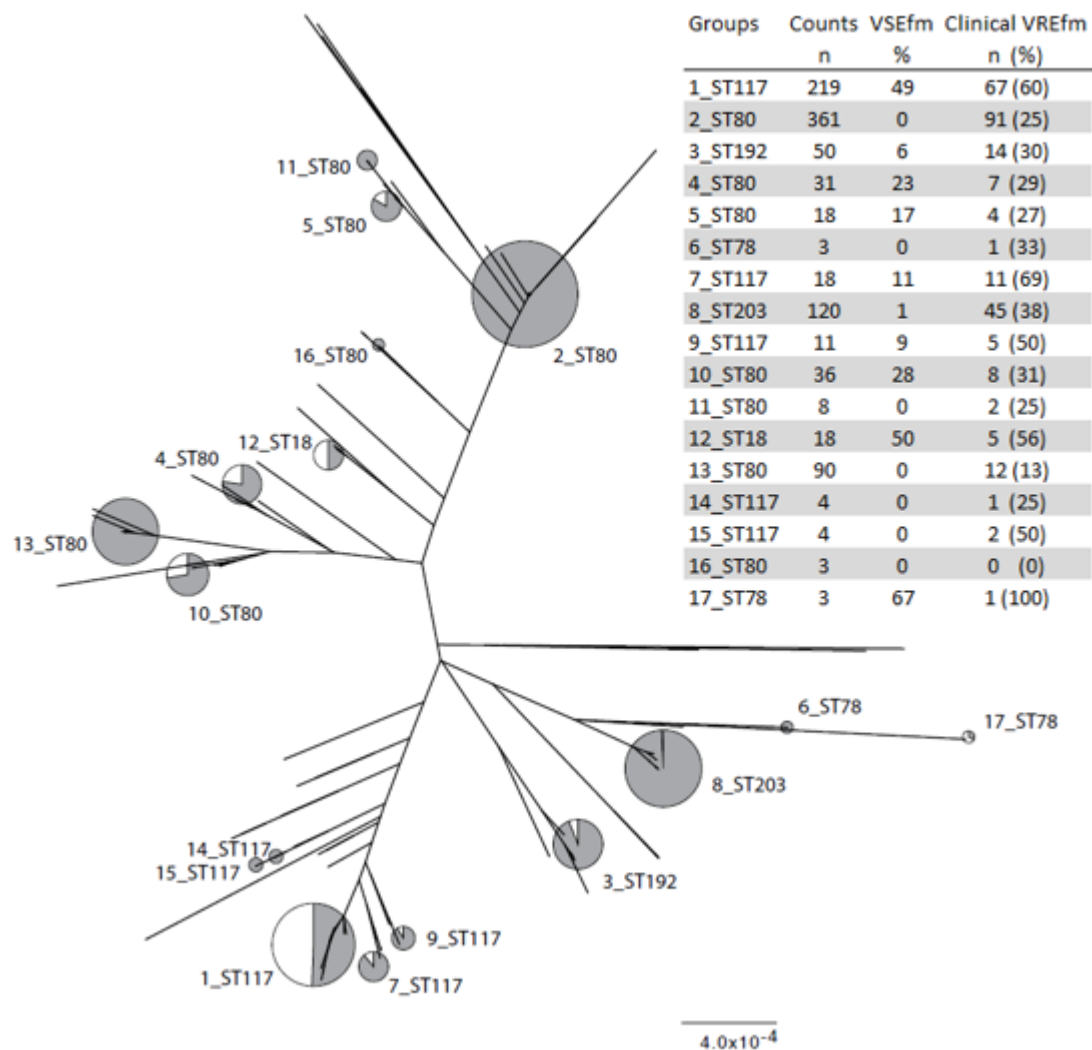
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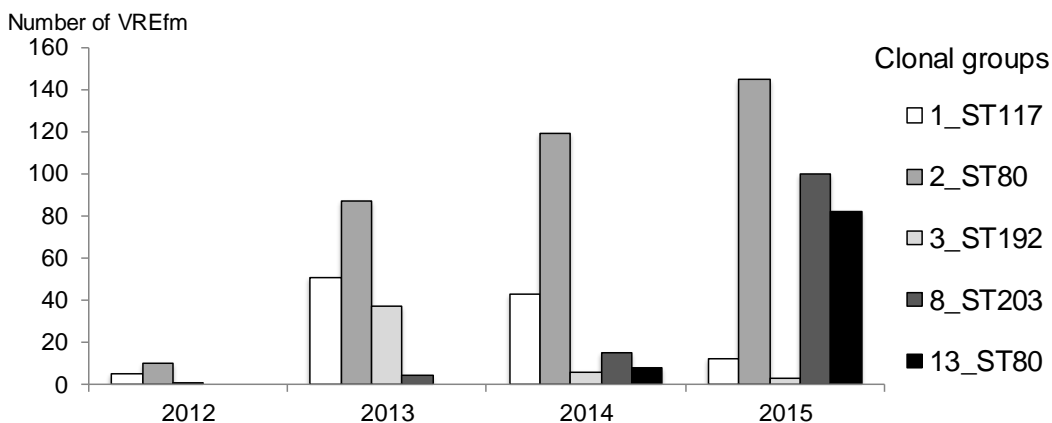


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563 A

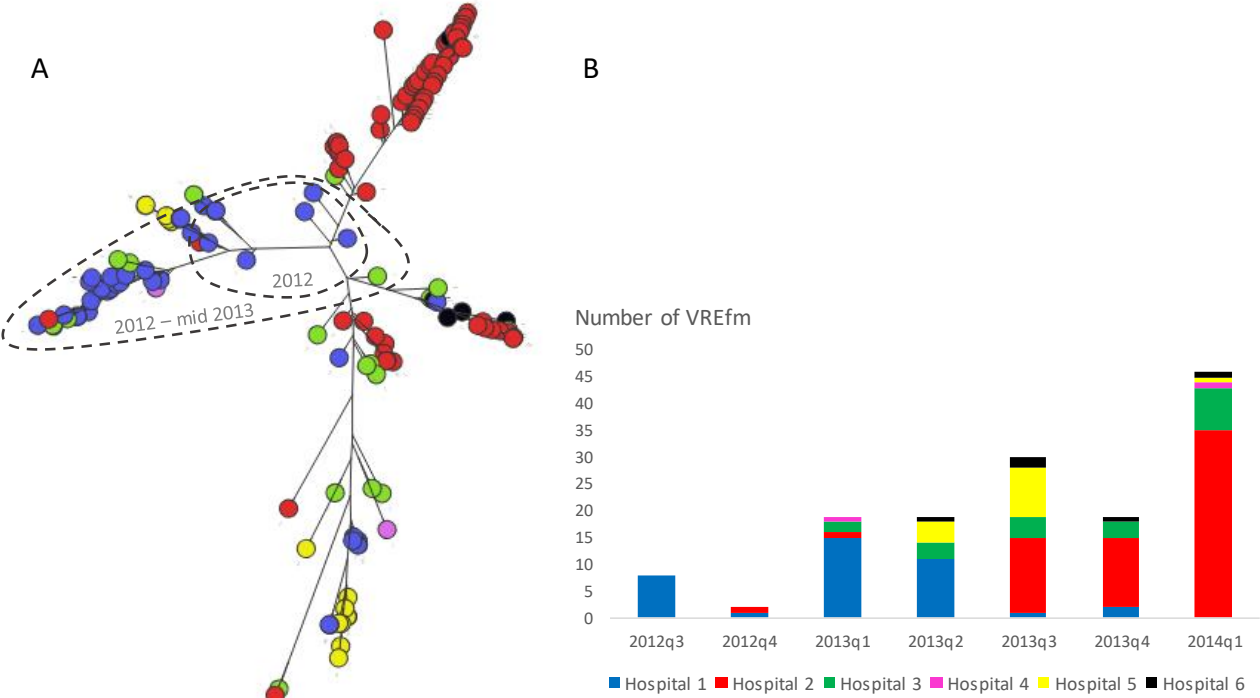


564 B



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566 Figure 1: A. SNP-based phylogeny of 892 *vanA* *E. faecium* (VREfm) and 166 vancomycin-susceptible *E.*  
567 *faecium* (VSEfm) identified in the Capital Region of Denmark from 2012-2015. Eight outliers have been  
568 removed from the phylogenetic tree. Each circle represents a main group and was scaled in relation to the  
569 number of isolates in the group. The pie charts show the proportion of VREfm (grey) and VSEfm (white)  
570 isolates within each main group. Circles are located near the centre of the group on the phylogenetic tree.

571 The group numbers are assigned according to when the group was first identified and according to ST.  
 572 Group identification number, number of isolates, percentage of VSEfm isolates in each main group and  
 573 percentage of clinical VREfm out of all VREfm are given in the table. B. The number of VREfm  
 574 belonging to the five largest clonal groups divided by calendar years.  
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578  
 579 Figure 2: A. Phylogenetic tree of clonal group 2\_ST80, 2012- March 2014 with masking of  
 580 recombination. Coloured circles represent the hospital origin of each of the isolates. The nested circular  
 581 shapes represent the approx. sampling times with inner circle contains isolates sampled until December  
 582 2012 and the outer circle contains isolates sampled until June 2013. Isolates outside the circles are  
 583 sampled from July 2013 to March 2014. Data are presented interactively at  
 584 <https://microreact.org/project/BkNqA1EDX> (use Google Chrome as browser).<sup>36</sup> B. Epidemic curve of  
 585 clonal group 2\_ST80, 2012- March 2014. This figure appears in colour in the online version of *JAC* and  
 586 in black and white in the printed version of *JAC*.  
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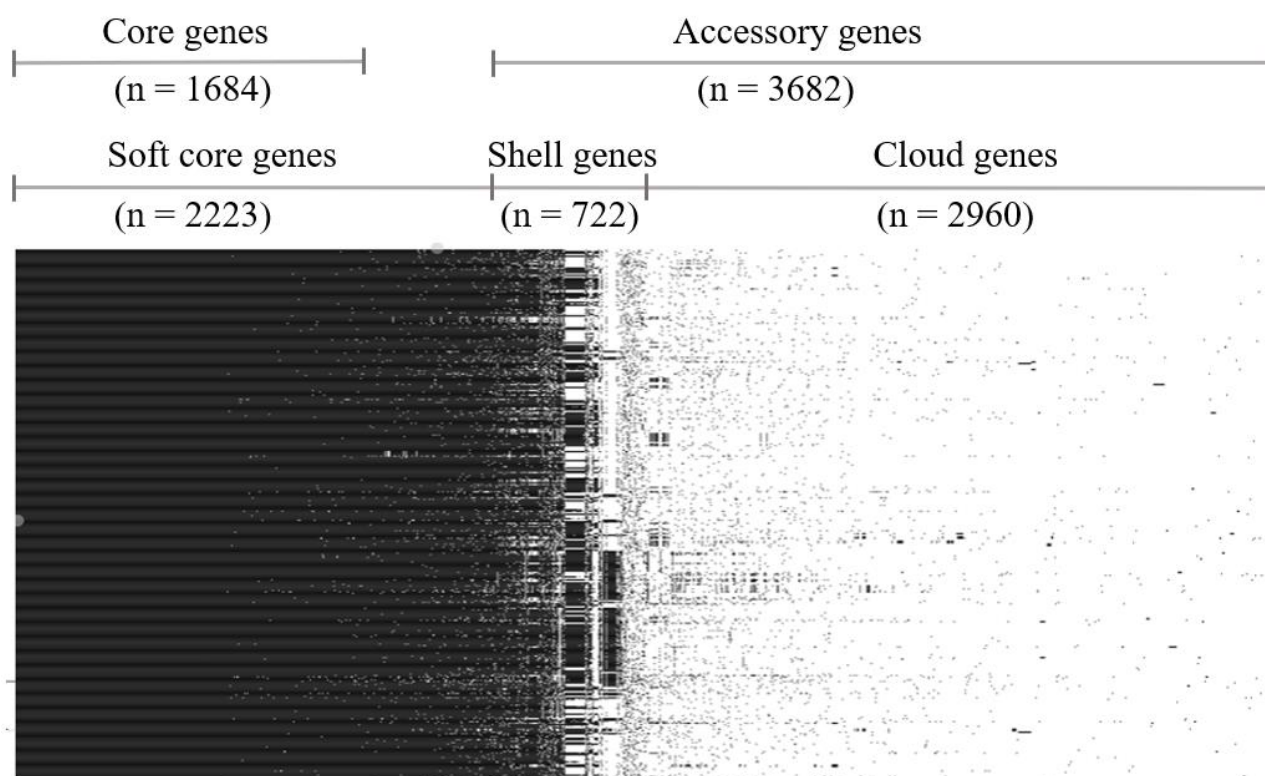


Figure 3: Visualization of the pan-genome of group 2\_ST80 (n=334 isolates). Each line represents an isolate. Presence of a gene is indicated in black and absence of a gene in white. Core genes (present in  $\geq 99\%$  of the isolates), soft core genes (present in  $\geq 95\%$  of the isolates), accessory genes (present in  $< 95\%$  of the isolates), shell genes (present in  $< 95\%$  and  $\geq 15\%$  of the isolates) and cloud genes (present in  $< 15\%$  of the isolates) are indicated. The isolates are vertically ordered based on similarity in a NJ-tree.

Genome	Size (bp)	GC content (%)	CDS	Circularisable	Copy number	Virulence genes	Resistance Genes	rep types	Prophage regions	Other features	Blast search in NCBI
Chromosome	2,720,495	38.2	2,625	Yes	1	<i>Acm</i>	<i>msrC</i> , <i>dfrG</i> , <i>tetM</i>		2 intact (28.7 kb, 35.9 kb), 1 questionable (14.8 kb) and 1 incomplete (18 kb)		
pV24-1	172,811	35.3	178	Yes	1	<i>HylEfm</i>	<i>Aac6_Aph2</i>	15	1 intact (24.8 kb)	<i>traG</i>	Ef AUS00233, coverage 73%, 99% identical
pV24-2	103,806	33.4	133	No	3				3 incomplete (7.9 kb, 10.8 kb, 11.6 kb)		Ef 6E6 p1, coverage 15%, 99% identical
pV24-3	61,681	33.0	70	Yes	2					<i>traG</i>	Ef pZB18 DNA, coverage 90%, 99% identical
pV24-4	41,792	35.4	47	Yes	3		<i>aph(3')-III</i> , <i>ant(6)-Ia</i> , <i>sat4A</i> , <i>tetS</i>	2	1 intact (24.5 kb)		Ef p5753cB, partial sequence, coverage 67%, 99% identical
pV24-5	41,388	35.4	51	Yes	3		<i>aph(3')-III</i> , <i>ant(6)-Ia</i> , <i>sat4A</i> , <i>ermB</i> , <i>vanA</i>	17	1 incomplete (24.5 kb)	<i>Txe-axe</i>	Ef pS177, coverage 88%, 99% identical
pV24-6	9,317	30.8	12	Yes	3					Bacteriocin	Ef AUS0085 p4, coverage 100%, 100% identical
pV24-7	6,173	35.5	8	Yes	6			11		Bacteriocin	Ef pB82, coverage 100%, 100% identical
pV24-8	5,954	33.3	6	Yes	7			18			Ef pVEF4, coverage 61%, 91% identical
pV24-9	4,465	32.1	4	Yes	5						Ef pHY DNA, coverage 61%, 99% identical
pV24-10	4,304	37.1	5	Yes	10						Ef AUS0004 p2, coverage 82%, 97% identical

Table 1: Summary of the *vanA E. faecium* genome of V24.

CDS: coding sequences, *Acm*: collagen adhesion gene, *HylEfm*: Hyaluronidase gene, *Aac6\_Aph2*, *aph(3')-III*, *ant(6)-Ia* and *sat4A*: resistance genes of aminoglycosides, *vanA*: resistance genotype of vancomycin, *msrC* and *ermB*: resistance genes of lincosamides, *dfrG*: resistance gene of trimethoprim, *tetS* and *tetM*: resistance genes of tetracyclin, *traG*: transfer gene, Txe-axe: Toxin-antitoxin system, Ef: *E. faecium*.

Group	n	pV24-1	pV24-2	pV24-3	pV24-4	pV24-5	pV24-6	pV24-7	pV24-8	pV24-9	pV24-10
----- n (%) -----											
1_ST117	111	4 (4)	98 (88)	0 (0)	1 (1)	99 (89)	5 (5)	108 (97)	107 (96)	29 (26)	21 (19)
2_ST80	361	15 (4)	227 (63)	103 (29)	167 (46)	357 (99)	29 (8)	359 (99)	350 (97)	340 (94)	257 (71)
3_ST192	47	43 (91)	12 (26)	0 (0)	0 (0)	44 (94)	1 (2)	42 (89)	42 (89)	45 (96)	42 (89)
4_ST80	24	2 (8)	12 (50)	0 (0)	1 (4)	19 (79)	0 (0)	15 (63)	17 (71)	19 (79)	15 (63)
5_ST80	15	0 (0)	13 (87)	0 (0)	0 (0)	15 (100)	0 (0)	14 (93)	4 (27)	2 (13)	3 (20)
7_ST117	16	0 (0)	1 (6)	0 (0)	1 (6)	15 (94)	0 (0)	16 (100)	16 (100)	2 (13)	2 (13)
8_ST203	119	1 (1)	37 (31)	0 (0)	1 (1)	1 (1)	0 (0)	118 (99)	1 (1)	8 (7)	4 (3)
9_ST117	10	10 (100)	9 (90)	0 (0)	0 (0)	7 (70)	0 (0)	10 (100)	10 (100)	3 (30)	3 (30)
10_ST80	26	0 (0)	11 (42)	0 (0)	0 (0)	20 (77)	0 (0)	26 (100)	13 (50)	26 (100)	13 (50)
11_ST80	8	0 (0)	8 (100)	0 (0)	0 (0)	8 (100)	0 (0)	8 (100)	8 (100)	0 (0)	0 (0)
12_ST18	9	2 (22)	5 (56)	0 (0)	0 (0)	4 (44)	0 (0)	3 (33)	3 (33)	2 (22)	1 (11)
13_ST80	90	84 (93)	46 (51)	0 (0)	1 (1)	89 (99)	0 (0)	90 (100)	89 (99)	90 (100)	61 (68)
Others	56	20 (36)	33 (59)	2 (4)	3 (5)	45 (80)	8 (14)	46 (82)	44 (79)	34 (61)	23 (41)
Total VREfm	892	181 (20)	512 (57)	105 (12)	175 (20)	723 (81)	43 (5)	854 (96)	704 (79)	600 (67)	444 (50)
Total VSEfm	166	16 (10)	33 (20)	0 (0)	0 (0)	0 (0)	12 (7)	123 (74)	116 (70)	31 (19)	14 (8)

Table 2: Plasmid distribution (pV24-1 to pV24-10) among vancomycin-resistant *E. faecium* (VREfm), vancomycin-susceptible *E. faecium* (VSEfm) and the 12 largest VREfm clonal groups.